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Resolution of 1,1,1-trifluoro-2-octanol by *Pseudomonas* sp. lipase encapsulated in aggregated silica nanoparticles†

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Lipase from *pseudomonas* sp. (PSL) was encapsulated in stacked silica nanoparticles and used for resolution of 1,1,1-trifluoro-2-octanol. The effects of reaction conditions, such as solvent, temperature, water activity and type of acyl donor were investigated. Compared with the free lipase, the encapsulated lipase exhibits better performance (enzyme activity, 20.32 μmol g^{-1} min^{-1}; *E* value, 33.83) under optimum conditions. Most importantly, almost no enzyme leakage phenomenon takes place during continuous batch operation, which suggests that this new encapsulation method has great potential for application.

Introduction

The approaches of enzyme immobilization can be broadly classified into three types: physical adsorption, covalent immobilization and encapsulation, each with its own advantages and disadvantages.[1–4] Physical adsorption is a simple, cheap and effective method, but enzyme leakage may occur due to the weak physical binding in most of the cases.[5–8] Covalent immobilization can efficiently avoid enzyme leakage, but it is complex and irreversible.[9–14] Encapsulation method can avoid the negative influence on the enzyme conformation, while the diffusion limitation of products or substrates is still a serious problem.[13,15–17]

Huo *et al.* originally synthesized a novel silica nanoparticle for delivering drug in blood pool.[18] To prevent the protein adsorption and platelet adhesion, the surface of the silica nanoparticle was modified by poly(ethylene oxide) (PEO) which could repel protein from the surface by steric repulsion and reduce the protein adsorption.[19] This kind of silica nanoparticle possesses good biocompatibility and dispersibility in water. Their results also demonstrate that the silica nanoparticles manifest its flexibility and mobility in the aqueous environment.

It's known that the cross-linking of silica species can take place during the drying process.[20] The lyophilization process can guarantee and accelerate the stack of silica nanoparticles under the stress of freezing and dehydration.[21] Once silica nanoparticles stack together, stacked silica nanoparticles can not disperse in water owing to the irreversible fusion of silica nanoparticles. In our previous work, we designed a new encapsulation method based on this phenomenon. After adding β-galactosidase into the silica nanoparticles solution, the enzyme was entrapped into the interstices formed by stacked nanoparticles during lyophilization. After lyophilization, most of β-galactosidase was entrapped into the interstices of silica nanoparticles matrices. Only a small quantity of enzyme was adsorbed on the surface of the matrices and could be easily removed by washing. Our research demonstrates that the thermal stability and pH stability of the encapsulated β-galactosidase in an aqueous environment are improved. In addition, the encapsulated β-galactosidase presents good reusability. However, this new encapsulation method has not yet been extensively studied in enzyme catalyzed resolution. Therefore more work in this area shall be done to widen its application.

In this study, we adopted the encapsulation method described above to immobilize lipase from *Pseudomonas* sp. (PSL). After encapsulation and characterization, encapsulated lipase was used for the resolution of 1,1,1-trifluoro-2-octanol. This reaction was a kinetically controlled transesterification. According to Kasche's report, the enzyme used in kinetically controlled synthesis should be withdrawn in time to obtain the maximum yield, and therefore the immobilization was the best strategy for solving this problem.[22] Rodrigues and coworker proposed that the yield of kinetically controlled synthesis depend on the enzyme performed in the reaction.[1] So, the effects of reaction conditions, such as solvent, temperature, water activity and type of acyl donor had been investigated. Furthermore, the reusability of encapsulated lipase had also been measured under its optimal reaction conditions.

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Experimental

Materials

Lipase from *Pseudomonas* sp. was kindly provided by Amano Enzymes Co. (Nagaya, Japan). Tetraethyl orthosilicate (TEOS), Pluronic F108 and dimethoxydimethylsilane ($Me_2Si(OMe)_2$, DMDMS) were commercially available from Sigma-Aldrich (St. Louis, Missouri, USA). KBr obtained from BDH Co. (Poole, UK) was of spectral grade. *R*-(+)-1-Phenylethyl isocyanate (*R*-(+)-PEIC) was purchased from Fluka (USA, 97%). 1,3,5-Trimethylbenzene (TMB) was purchased from Tianjin Guangfu Fine Chemical Research Institute of China. 1,1,1-Trifluoro-2-octanol, vinyl esters and other organic solvents of analytical grade were purchased from Shanghai Chemical Reagent Company (China). All other chemicals and reagents were of analytical grade. All aqueous solutions were prepared with Milli-Q water.

Synthesis of silica cross-linked micellar nanoparticle

In a typical preparation, the synthesis of silica nanoparticles was achieved by the previous method with a slight modification.[23] Pluronic F108 (0.1 g) was dissolved in 10 g of HCl solution (2 M) with stirring at room temperature, followed by the addition of TMB (60 μl). The mixture was stirred at room temperature for 0.5 hour. Then 0.33 g of TEOS was added to the above solution. The mixture was stirred at room temperature for 0.5 hour again, and then 0.08 g of DMDMS was added. Stirring was continued at room temperature for 0.5 hour.

The HCl and EtOH (resulted from the hydrolysis of TEOS and DMDMS) were removed by dialysis (Millipore, MW cutoff 8000–12 000) against pure water with stirring at room temperature. The water was refreshed every 2 hours. The dialysis process was monitored by measuring the pH of solution and terminated at pH 7.0. Then, the silica nanoparticle solution (40 mg ml^{-1}) was prepared.

Encapsulating the lipase

Crude lipase was purified as follows: 2 g of crude lipase was dissolved in 10 ml of PBS buffer (50 mM, pH 7.0) in ice bath. The lipase solution was centrifuged at 3500*g* for 3 hours using ultrafiltration device (Millipore, MW cutoff 10 000). The solution above the membrane was collected and then lyophilized. The lipase solution (8 mg ml^{-1}) was prepared by redissolving the lyophilized lipase in the buffer at 4 °C.

The lipase solution (5 ml, 8 mg ml^{-1}) was slowly added to 10 ml of the silica nanoparticle solution under stirring at 4 °C. The resulting solution was frozen at −20 °C for 30 min and then lyophilized. The lyophilized product was repeatedly rinsed with Milli-Q water at room temperature, until no absorbance of protein in supernatant could be detected. Wet sample was lyophilized again. Finally, lyophilized powder was stored at −20 °C.

The protein content of enzyme solutions was determined by Bradford method.[24] Bovine serum albumin was used as standard. The encapsulation yield (the amount ratio of entrapped lipase to lipase added to the encapsulated system) of lipase was 93.8% in this experiment.

Characterization of silica nanoparticle

Transmission electron microscopy was performed on a FEI Tecnai G2 F20 s-twin (FEI, American) at 200 keV. The particle size of the silica nanoparticles was measured by a Malvern Zetasizer Nano-S instrument at room temperature using Dynamic Light Scattering (DLS) principle with a HeNe laser (633 nm). FTIR spectrum was surveyed using Nicolet 5700 FTIR spectrometer with a resolution of 4 cm^{-1} through the KBr method.

Resolution of 1,1,1-trifluoro-2-octanol catalyzed by the encapsulated lipase

The reaction was implemented in a round bottom flask contained 1,1,1-trifluoro-2-octanol (2 mmol), vinyl acetate (5 mmol), *n*-hexane (10 ml), water activity ($a_w = 0.58$) and the encapsulated lipase (50 mg) at 50 °C.

Water activity (a_w) control and measurement

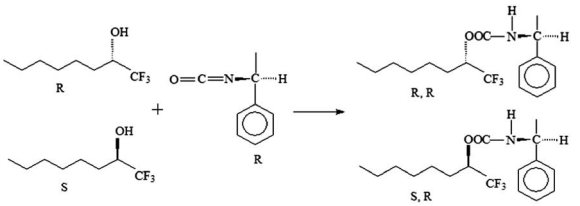
Water activity (a_w) was controlled according to the method described by Tian.[25] All the reaction mixture components were previously dried in a vacuum of 1 mmHg for 12 h. Following that, all reaction mixtures with specific water activity (a_w) were prepared by adding a specific amount of water. The resulting samples were pre-equilibrated at desired temperature for 24 h in a sealed vial before being subjected to a_w measurement. Water activity (a_w) was measured by Hygrolab Humidity Detector (Rotronic, Switzerland).

Reusability

The encapsulated lipase was used for successive batches. After each cycle, reaction mixture was centrifuged at 12 000 rpm for 3 min. The obtained precipitate was washed three times with *n*-hexane to remove any residual substrate or product and then kept overnight in a vacuum oven for a complete drying. Dried enzyme powder was used at next cycle under otherwise equivalent conditions. The residual activity of recycled enzyme was compared with the enzyme activity of the first cycle (100%).

Determination of enantiomeric excess and *E* value

The enantiomeric excess and *E* value of encapsulated lipase was measured according to previous method described by Yu.[26] Samples were withdrawn from the vials and analyzed directly by gas chromatograph on a Shimadzu gas chromatograph (GC-14B) equipped with a FID detector and a column (EC-1000, 30 m × 0.25 mm × 0.25 μm, Alltech). The temperatures of injector and detector were 200 and 290 °C, respectively. Nitrogen was used as carrier gas at a flow rate of 60 ml min^{-1}. Temperature programming between 110 and 210 °C with the increment of 15 °C min^{-1} was used to determine the concentration of 1,1,1-trifluoro-2-octanol. (*S*)-1,1,1-Trifluoro-2-octanol or (*R*)-1,1,1-trifluoro-2-octanol was derived from *R*-(+)-PEIC (Scheme 1). The distinction of *R* from *S* enantiomers was achieved with temperature programming between 110 and 222 °C with the increment of 10 °C min^{-1}.



Scheme 1 The formation of isomer for pre-column derivation.

The conversion (C) was determined *via* the decrease of 1,1,1-trifluoro-2-octanol. Enzyme activity (μmol min^{-1} g^{-1}) was defined as the amount (in micromoles) of 1,1,1-trifluoro-2-octanol ester produced per minute per gram of encapsulated enzyme. The enantiomeric excess of 1,1,1-trifluoro-2-octanol (ee_S) was determined by calculating the peak areas of two derivatives eqn (1). The enantiomeric ratio (E value) was determined from C and ee_S by using eqn (2).[27] The enzyme activity and the E value of the enzyme were the average of three data points. All the three data points were detected and calculated at about 20% of conversion (in the range of 15–30%).

$$ee_s(\%) = \frac{[S-R]}{[S+R]} \times 100 \quad (1)$$

$$E \text{ value } E = \frac{\ln[(1-c)(1-ee_s)]}{\ln[(1-c)(1+ee_s)]} \quad (2)$$

where S and R represent the concentrations of the (S,R)-diastereomer and (R,R)-diastereomer, respectively.

Results and discussion

In this study, the silica nanoparticles were firstly synthesized. Then, PSL was encapsulated in the interstices formed by stacked silica nanoparticles during lyophilization (Fig. 1).

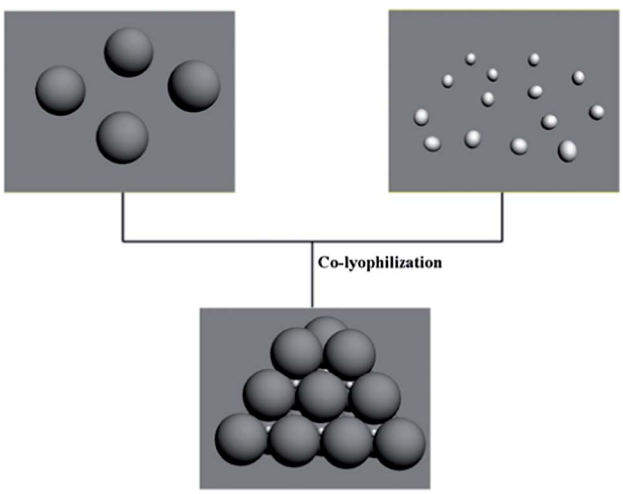


Fig. 1 The encapsulation strategy for entrapping lipase in the interstices of stacked silica nanoparticles. The black sphere and the white ellipsoid represent silica nanoparticle and lipase, respectively.

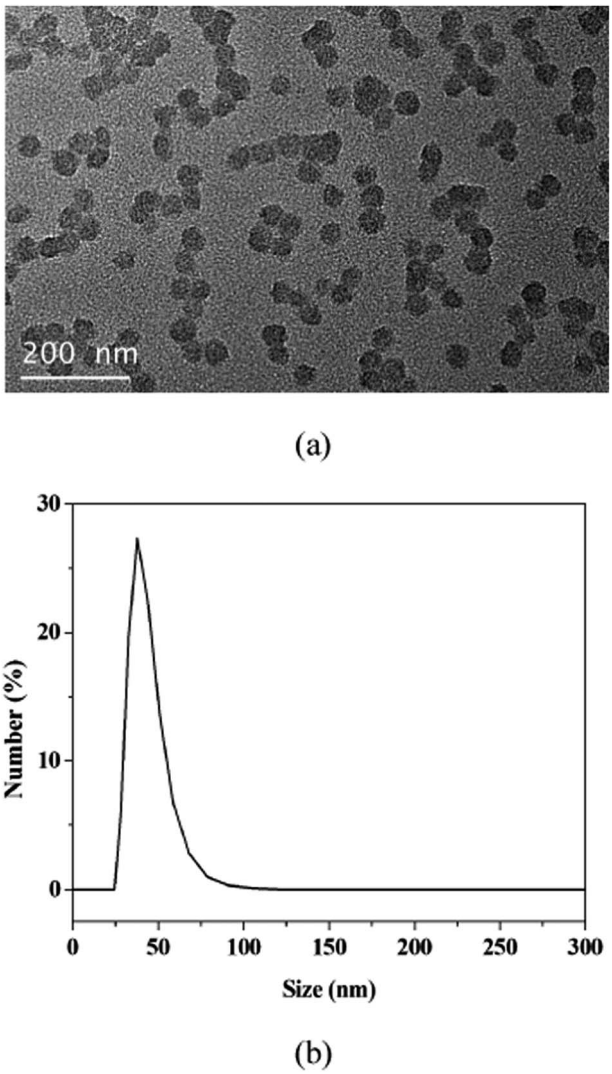


Fig. 2 TEM images (a) and light scattering measurement (b) of the synthesized silica nanoparticles.

Synthesis and assembly of silica nanoparticles

In this experiment, the silica nanoparticles were synthesized according to our previous method with a slight modification.[28] The silica nanoparticles were characterized by TEM (Fig. 2a) and light scattering measurement (Fig. 2b). It can be found that a uniform spherical nanoparticle is obtained and its average diameter is about 45 nm.

The lyophilization can remove the water from the nanoparticles solution and guide the silica nanoparticles to stack together. So, in this study, PSL was encapsulated in the matrices formed by stacked silica nanoparticle through lyophilization. After encapsulation and washing, immobilized enzyme was characterized by TEM and FTIR, respectively.

TEM image of immobilized sample demonstrates that aggregated silica nanoparticles are formed after lyophilization (Fig. 3). FTIR spectrum of the silica nanoparticles, the lipase and the encapsulated lipase are shown in Fig. 4. The stretching vibration band of Si–O–Si can be found at 1100 cm^{-1} (curve 1

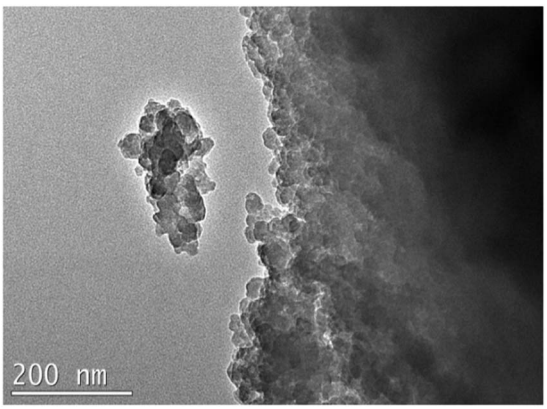


Fig. 3 The TEM of the aggregated silica nanoparticles loaded with the lipase.

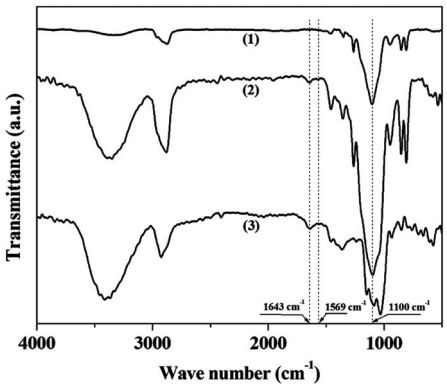


Fig. 4 FTIR spectrum of the aggregation of the silica nanoparticles (1), the aggregation of the silica nanoparticles loading with the lipase (2) and pure lipase (3).

and curve 2 in Fig. 4). And the amide I and II bands of the lipase can be observed at 1643 cm^{-1} and 1569 cm^{-1} (curve 2 and curve 3 in Fig. 4).[29] Moreover, our results verify that the aggregated silica nanoparticles can not strongly adsorb the lipase (see ESI†) The FTIR result demonstrates that the lyophilized product is composed by silica-based matrices and the protein. These results demonstrates that lipase from *Pseudomonas* sp. has been successfully encapsulated in interstices formed by stacked silica nanoparticles after lyophilization.

The effect of mass ratio (silica nanoparticles/lipase) on the encapsulation yield

The encapsulation yield depends on the mass ratio (silica nanoparticles/lipase) used in this experiment. The mass ratio in the range of 2–20 was investigated to obtain optimal encapsulation yield (Fig. 5). The experimental result exhibits that the encapsulation yield dramatically increases as the mass ratio increases in the range of 2–10. With the further increase of the mass ratio in the range of 10–20, the encapsulation yield reaches a plateau. This result verifies that almost all the lipase can be encapsulated in stacked silica nanoparticles when the mass ratio is above 10. Hence, the mass ratio of 10 was chosen in the following experiment.

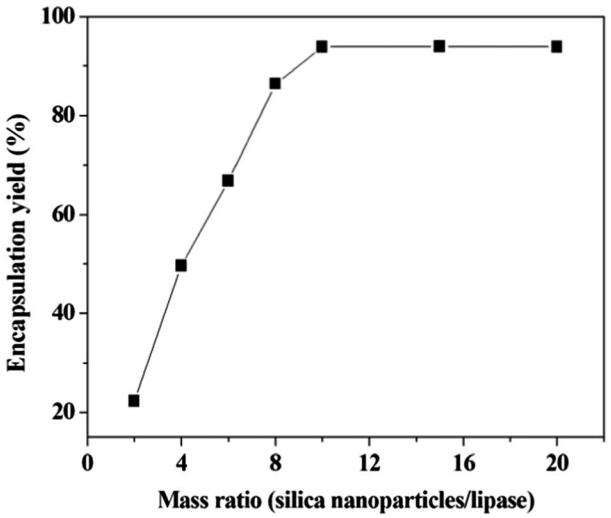


Fig. 5 Effect of mass ratio (silica nanoparticles/lipase) on the encapsulation yield.

Optimizing the condition for resolution of 1,1,1-trifluoro-2-octanol

Temperature is an important parameter for an enzyme-catalyzed resolution reaction.[30] In this study, the relationship

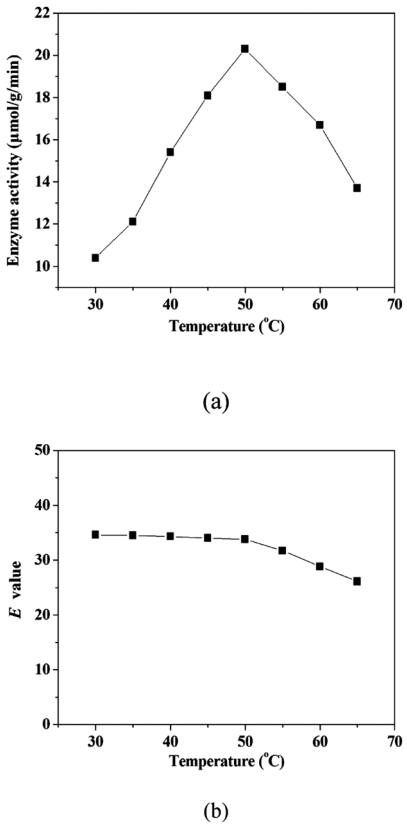


Fig. 6 Effect of temperature on the enzyme activity (a) and the enantioselectivity (b) of the encapsulated lipase in resolution of 1,1,1-trifluoro-2-octanol. Reactions were carried out in *n*-hexane (10 ml) with 1,1,1-trifluoro-2-octanol (2 mmol), vinyl acetate (5 mmol), the encapsulated lipase (50 mg) and water activity (0.58) at various temperatures (30–65 °C).

Table 1 Effect of solvents on the activity and enantioselectivity of the encapsulated lipase in resolution of 1,1,1-trifluoro-2-octanol[a]

Solvent	log *P*	Enzyme activity (μmol g^{-1} min^{-1})	*E* value
n-Heptane	4.60	18.88	32.86
n-Hexane	3.50	20.32	33.83
Toluene	2.50	17.20	30.20
Cyclohexane	1.20	14.04	27.03
Acetonitrile	−0.33	5.58	10.49
DMF	−1.00	2.78	5.17
1,4-Dioxane	−1.10	1.60	4.20

[a] Reactions were carried out in different solvents (10 ml) with 1,1,1-trifluoro-2-octanol (2 mmol), vinyl acetate (5 mmol), the encapsulated lipase (50 mg) and water activity (0.58) at 50 °C.

between temperature and enzyme performance of encapsulated lipase in resolution of 1,1,1-trifluoro-2-octanol was examined in the range of 30–65 °C. It can be found that enzyme activity exhibits a bell shaped curve with changing temperature and maximum enzyme activity is observed at 50 °C (Fig. 6a). When considering enantioselectivity, it can be found that *E* value continuously decreases with the increase of temperature (Fig. 6b), which is in agreement with Phillips' viewpoint that the highest enantioselectivity can be gained at low temperature due to enthalpic control.[30] Because encapsulated lipase has the highest activity and a good enantioselectivity at 50 °C, 50 °C was selected as optimal reaction temperature for further study.

For non-aqueous enzymology, the enzyme performance is often affected by the polarity of organic solvents.[31] In this study, the activity and enantioselectivity of encapsulated lipase in organic solvents with different log *P* (−1.1–4.6) were investigated to optimize the reaction condition. As shown in Table 1, the activity and enantioselectivity of the encapsulated lipase increase with the increase of log *P*. Generally speaking, the hydrophilic solvent may disrupt optimal enzyme conformation and affect enzyme performance by interacting with the active site of enzyme directly or stripping off the essential water from enzyme.[32] In this study, the highest enzyme activity and enantioselectivity of encapsulated lipase were obtained when *n*-hexane was used as the organic media.

We also screened the optimum acyl donor in this study and the results were listed in Table 2. The highest enantioselectivity of encapsulated lipase was obtained when vinyl butyrate was used as acyl donor. Enzyme activity is the highest when vinyl acetate is used as acyl donor and it becomes lower when the carbon chain length of acyl donors increases. Considering that vinyl acetate is the cheapest acyl donor and it possesses the lowest boiling point, which is helpful for its removing from reaction system, vinyl acetate was selected as optimal acyl donor in this study.

Table 2 Effect of acyl donor on enantioselectivity transesterification[a]

Acyl donor	Enzyme activity (μmol g^{-1} min^{-1})	*E* value
Vinyl acetate	20.32	33.83
Vinyl propionate	14.72	42.84
Vinyl butyrate	11.25	43.88
Vinyl valerate	8.90	40.40
Vinyl caproate	6.73	35.43

[a] Reactions were carried out in *n*-hexane (10 ml) with 1,1,1-trifluoro-2-octanol (2 mmol), the encapsulated lipase (50 mg), water activity (0.58) and different acyl donors (5 mmol) at 50 °C.

The hydration level of enzyme dominates protein flexibility and then influences enzyme performance. The amount of water associated with enzyme can be controlled by water activity.[33] As shown in Fig. 7a, the enzyme activity gradually increases with increasing a_w in the range of 0.04–0.58, and then decreasing sharply from a_w 0.58 to 0.91. At low a_w (0.04–0.13), low enzyme activity is observed owing to the absence of "necessary water" on the surface of lipase which contributes to form favorable conformation of enzyme for achieving transesterification reaction.[32] At moderate a_w values (0.13–0.58), water allows sufficient enzyme flexibility for catalytic activity. Along with the further increase of a_w (0.58–0.91), the aggregation between lipases may occur, which shall block the access of active site of lipase for 1,1,1-trifluoro-2-octanol so as to reduce enzyme activity. Another possible explanation is as follows: when the "necessary water" of lipase approaches to saturation, surplus water acting as competing nucleophile attack acyl–enzyme complex to

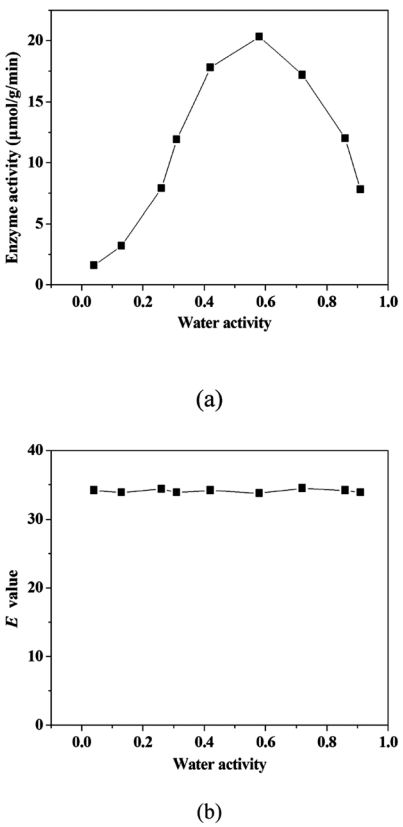


Fig. 7 Effect of water activity on the enzyme activity (a) and the enantioselectivity (b) of the encapsulated lipase in resolution of 1,1,1-trifluoro-2-octanol. Reactions were carried out in *n*-hexane (10 ml) with 1,1,1-trifluoro-2-octanol (2 mmol), vinyl acetate (5 mmol), the encapsulated lipase (50 mg) and water activity (0.04–0.91) at 50 °C.

Table 3 Resolution of 1,1,1-trifluoro-2-octanol in repeated batch process by encapsulated lipase in the aggregated silica nanoparticles[a]

Batch	Enzyme activity (μmol g^{-1} min^{-1})	*E* value
1	20.32	33.83
2	20.32	33.83
3	20.31	33.50
4	20.23	33.34
5	20.18	33.17

[a] Reactions were carried out in *n*-hexane (10 ml) with 1,1,1-trifluoro-2-octanol (2 mmol), vinyl acetate (5 mmol), the encapsulated lipase (50 mg) and water activity (0.58) at 50 °C.

inhibit acyl transfer reactions. The results also demonstrate that *E* value is not significantly influenced by the changes of a_w (Fig. 7b), which is similar to the results in published literature.[34]

Reusability

It's known that one of the best advantages of the immobilized enzyme is its low cost in practical application by reuse. Therefore, the reusability of the encapsulated lipase deserves further research. As shown in Table 3, the encapsulated lipase remains 99.5% of its initial activity as well as 98.2% of initial enantioselectivity even after five runs. Furthermore, almost no leakage phenomenon has been detected during continuous batch operations. The perfect performance may attribute to the size matching between the lipase and the interstices in aggregated silica nanoparticles matrices.

Free lyophilized lipase *vs.* the encapsulated lipase

As shown in Table 4, the enzyme activity and *E* value of encapsulated lipase were 1.5 and 2 times as much as that of free lipase, respectively. It's known that most lipases can show interfacial activation which induce the appearance of the active open-lid conformation.[35] Furthermore, as suggested by Roberto and coworkers, bimolecular structure of lipase from *Pseudomonas fluorescens* (PFL) can also enhance the enzyme activity by mutual interfacial activation.[36] However, if larger enzyme aggregation forms, the enzyme activity shall be inhibited. In this study, PSL was encapsulated into the small interstice of stacked silica nanoparticles after lyophilization, which can reduce the larger enzyme aggregation. Furthermore, the interaction between lipase and silica nanoparticles becomes inevitable. The hydrophobic interactions between hydrophobic area of lipase and the hydrophobic group of the matrices may cause interfacial activation of encapsulated lipase which can induce the formation of the active open-lid conformation and increase the enzyme performance of lipase. The hydrophobic group of the matrices mainly originates from the methyl group of dimethoxydimethylsilane.

Table 4 Enzyme activity and *E* value of lyophilized and encapsulated lipase[a]

Enzyme	Enzyme activity (μmol g^{-1} min^{-1})	*E* value
Free lipase	13.48	16.87
Encapsulated lipase	20.32	33.83

[a] For encapsulated enzyme, the experimental data came from the results of reusability; for lyophilized lipase, reactions were carried out in *n*-hexane (10 ml) with 1,1,1-trifluoro-2-octanol (2 mmol), vinyl acetate (5 mmol), lyophilized lipase (50 mg) and water activity (0.58) at 50 °C.

Conclusions

In this study, the lipase from *Pseudomonas* sp. was encapsulated into the aggregated silica nanoparticles by lyophilization and successfully used for resolution of 1,1,1-trifluoro-2-octanol. Almost no leakage phenomenon takes place during continuous batch operations. Furthermore, these high-quality silica nanoparticles can be easily synthesized. These results prove that the new encapsulation method has a great potential for application.

Acknowledgements

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